PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 16/28, 16/46, 14/05, 14/745, A61K 39/395 — A1

(11) International Publication Number: WO 96/12742

(43) International Publication Date: 2 May 1996 (02.05.96)

(21) International Application Number: PCT/EP95/04110

(22) International Filing Date: 20 October 1995 (20.10.95)

(30) Priority Data:

9421463.2	25 October 1994 (25.10.94)	GB
9512480.6	20 June 1995 (20.06.95)	GB
9513415.1	30 June 1995 (30.06.95)	GB

(71) Applicant *(for all designated States except US)*: GLAXO GROUP LIMITED [GB/GB]; Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BONNEFOY, Jean-Yves, Marcel, Paul [FR/CH]; The Glaxo Institute of Molecular Biology, 14, chemin des Aulx, Case postale 674, CH-1228 Plan-les-Ouates (CH). LECOANET-HENCHOZ, Sybille [CH/CH]; The Glaxo Institute of Molecular Biology, 14, chemin des Aulx, Case postale 674, CH-1228 Plan-les-Ouates (CH).

(74) Agent: DAWSON, Hugh, B.; Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).

(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BINDING AGENTS FOR TREATMENT OF INFLAMMATORY, AUTOIMMUNE OR ALLERGIC DISEASES

(57) Abstract

Binding agents to CD11b, CD11c, CD21, CD23, a 70 to 85 KDa protein expressed on endothelial cells or a 115 KDa protein expressed on endothelial cells, can be useful in the treatment of inflammatory, autoimmune or allergic disease.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

BINDING AGENTS FOR TREATMENT OF INFLAMMATORY, AUTOIMMUNE OR ALLERGIC DISEASES

The present invention relates to particular binding agents which can be used in the treatment of inflammatory, autoimmune or allergic diseases.

CD23 (FCεRII) is a type II molecule of the C-lectin family which also includes the lymphocyte homing receptor (MEL-14) and the endothelial leukocyte adhesion molecule-1 (ELAM-1). It is a low affinity receptor for IgE. In humans a variety of haematopoietic cell types express CD23 on their surface, including follicular dendritic cells, B cells, T cells and macrophages. CD23 molecules are also found in soluble forms in biological fluids. Soluble CD23 (sCD23) molecules are formed by proteolytic cleavage of transmembrane receptors. CD23 has pleiotropic activities including mediation of cell adhesion, regulation of IgE and histamine release, rescue of B cells from apoptosis and regulation of myeloid cell growth. These functional activities are mediated through the binding to specific ligands of cell-associated CD23, or sCD23, the latter acting in a cytokine-like manner (Conrad, D.H., *Annu Rev Immunol* **8**, 623-645 1990); Delespesse, G., *et al.*, *Adv Immunol 49*, 149-191 (1991); Bonnefoy, J.Y., *et al.*, *Curr Opin Immunol* **5**, 944-947 (1993)).

Increased expression of CD23 has been observed in a number of inflammatory diseases. CD23 has been identified in synovial biopsies from patients with chronic synovitis, and sCD23 can be measured at concentrations exceeding the normal range in the serum and synovial fluid of patients with rheumatoid arthritis (Bansal, A.S., Oliver, W., Marsh, M.N., Pumphrey, R.S., and Wilson, P.B., *Immunology* **79**, 285-289 (1993); Hellen, E.A., Rowlands, D.C., Hansel, T.T., Kitas, G.D., and Crocker, J.J., *Clin Pathol* **44**, 293-296 (1991); Chomarat, P., Brioloay, J., Banchereau, J., & Miossec, P., *Arthritis Rheum* **86**, 234-242 (1993); Bansal, A., *et al.*, *Clin Exp Immunol* **89**, 452-455 (1992); Rezonzew, R., & Newkirk, M.M., *Clin*

Immunol Immunopathol **71**, 156-163 (1994)). In addition, levels of serum sCD23 in rheumatoid arthritis patients are related to disease status and correlate with serum rheumatoid factor (Bansal, A.S., *et al.*, *Clin Exp Rheumatol* **12**, 281-285 (1994)). Pro-inflammatory cytokines appear to be particularly important in rheumatoid arthritis, and a central role for TNF-α and IL-1β in the destruction of arthritic joints has been postulated (Brennan, F.M., Chantry, D., Jackson, A., Maini, R., & Feldman, M., *Lancet* **2**, 244-247 (1989); Brennan, F.M., Maini, R.M., & Feldman M., *Br J Rheumatol* **31**, 293-298 (1992)).

It has also been postulated that CD23-CD21 interactions may play a role in the control of IgE production (Flores-Romo L. *et al.*, *Science* **261** 1038-1041 (1993); Aubry *et al.*, *Nature* **358**, 505-507 (1992)).

CD11b and CD11c are adhesion molecules that participate in many cell-cell and cell-matrix interactions. CD11b/CD18 and CD11c/CD18 (an association of CD11b and CD18 and of CD11c and CD18 respectively) have been reported to bind several ligands, including CD54, fibrinogen, factor X, LPS, Con A and zymosan (Springer, T.A, *Nature* **346**, 425-434 (1990)). The role of these binding molecules is not however completely understood. CD11b/CD18 and CD11c/CD18 are also known as MAC-1 and p150, 95 respectively. They are members of the β_2 integrin family (sometimes known as Leu-CAM, ie leucocyte cell adhesion molecules). This family also includes LFA-1 amongst its members (also known as CD11a/CD18).

EP 0205405 purports to disclose Mabs to lymphocyte cellular receptors for IgE (FCεR) cross reacting with human immunoglobulin E binding factor (IgE-BF), and derivatives thereof.

WO 93/04173 purports to disclose a polypeptide which is capable of binding to one of FCEL (low affinity IgE receptor FCεRII) or FCEH (high affinity receptor FCεRI) but which is substantially incapable of binding to the other of FCEL or FCEH. Treatment of an allergic disorder is alleged with a FCEL or FCEH specific polypeptide (provided the FCEH specific polypeptide is incapable of crosslinking FCEH and inducing histamine release).

EP 0269728 purports to disclose Mabs to the human lymphocyte IgE receptor.

EP 0259585 purports to disclose Mabs recognising a surface receptor for IgE (FCεR) on human B lymphocytes.

WO 93/02108 purports to disclose primatised antibodies for therapeutic use.

The present inventors have surprisingly discovered that binding agents to CD21, CD11b, CD11c, to a 70-85 KDa protein expressed on endothelial cells, or to a 115 KDa protein expressed on endothelial cells can be of utility in the treatment or prophylaxis of various diseases and in particular in the treatment or prophylaxis of arthritis. Prior to the present invention no data has been presented which would support such a utility, despite the publication of a large number of papers in which CD21, CD11b or CD11c have been discussed.

According to the present invention, there is provided a binding agent to CD21, CD11b, CD11c, to an 70-85 KDa protein expressed on endothelial cells, or to a 115 KDa protein expressed on endothelial cells for use in the treatment or prophylaxis of inflammatory, autoimmune or allergic diseases.

The binding agent may function by blocking the interaction between the protein and a ligand which binds to it. *In vitro* assays e.g. radio-immune assays may be used to study such a blocking effect.

The binding agent may be in isolated form or as part of a pharmaceutical composition. Desirably it is in sterile form. Generally speaking a binding agent which is specific for CD21, CD11b, CD11c, to a 70-85 KDa protein expressed on endothelial cells, (e.g. to a 76 kDa, a 80 KDa or 85 KDa protein expressed on endothelial cells) or to a 115 KDa protein expressed on endothelial cells, is useful in the treatment/prophylaxis disclosed.

Preferred binding agents include antibodies, fragments thereof or artificial constructs comprising antibodies or fragments thereof or artificial constructs designed to mimic the binding of antibodies or fragments thereof. Such binding agents are discussed by Dougall *et al* in *Tibtech* **12**, 372-379 (1994).

They include complete antibodies, $F(ab')_2$ fragments, Fab fragments, Fv fragments, ScFv fragments, other fragments, CDR peptides and mimetics. These can be obtained/ prepared by those skilled in the art. For example, enzyme digestion can be used to obtain $F(ab')_2$ and Fab fragments (by subjecting an IgG to molecule to pepsin or papain cleavage respectively). References to "antibodies" in the following description should be taken to include all of the possibilities mentioned above.

Recombinant antibodies may be used. The antibodies may be humanized; or chimaerised.

A typical preparation of a humanised antibody in which the CDRs are derived from a different speci s than the framework of the antibody's variabl domains is

disclosed in EP-A-0239400. The CDRs may be derived from a rat or mouse monoclonal antibody. The framework of the variable domains, and the constant domains, of the altered antibody may be derived from a human antibody. Such a humanised antibody elicits a negligible immune response when administered to a human compared to the immune response mounted by a human against a rat or mouse antibody.

Alternatively, the antibody may be a chimaeric antibody, for instance of the type described in WO 86/01533.

A chimaeric antibody according to WO 86/01533 comprises an antigen binding region and a non-immunoglobulin region. The antigen binding region is an antibody light chain variable domain or heavy chain variable domain. Typically, the chimaeric antibody comprises both light and heavy chain variable domains. The non-immunoglobulin region is fused at its C-terminus to the antigen binding region. The non-immunoglobulin region is typically a non-immunoglobulin protein and may be an enzyme region, a region derived from a protein having known binding specificity, from a protein toxin or indeed from any protein expressed by a gene. The two regions of the chimaeric antibody may be connected via a cleavable linker sequence.

The antibody may be a human IgG such as IgG1, IgG2, IgG3, IgG4; IgM; IgA; IgE or IgD carrying rat or mouse variable regions (chimaeric) or CDRs (humanised). Primatizing techniques may also be used, such as those disclosed in WO93/02108.

Other preferred binding agents (apart from antibodies or derivatives thereof) are Factor X (i.e. Factor 10), Epstein Barr Virus or a part of Epstein Barr Virus.

Where intact viruses are used in medical treatment, they will generally be provided in non-virulent form. This can be achieved using techniques known to those skilled in the art, which include attenuation and mutagenesis.

As will be appreciated by those skilled in the art, where specific binding agents are described herein, derivatives of such agents can also be used. The term "derivative" includes variants of the agents described, having one or more amino acid substitutions, deletions or insertions relative to said agents, whilst still having the binding activity described. Preferably these derivatives have substantial amino acid sequence identity with the binding agents specified.

The degree of amino acid sequence identity can be calculated using a program such as "bestfit" (Smith and Waterman, Advances in Applied Mathematics, 482-489 (1981)) to find the best segment of similarity between any two sequences. The alignment is based on maximising the score achieved using a matrix of amino acid similarities, such as that described by Schwarz and Dayhof (1979) Atlas of Protein Sequence and Structure, Dayhof, M.O., Ed pp 353-358.

Preferably the degree of sequence identity is at least 50% and more preferably it is at least 75%. Sequence identities of at least 90% or of at least 95% are most preferred.

It will nevertheless be appreciated by the skilled person that high degrees of sequence identity are not necessarily required since various amino acids may often be substituted for other amino acids which have similar properties without substantially altering or adversely affecting certain properties of a protein. These are sometimes referred to as "conservative" amino acid changes. Thus the amino acids glycine, valine, leucine or isoleucine can often be substituted for one another

(amino acids having aliphatic hydroxyl side chains). Other amino acids which can often be substituted for one another include:
phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains) and cysteine and methionine (amino acids having sulphur containing side chains). Thus the term "derivative" can also include a variant of an amino acid sequence comprising one or more such "conservative" changes relative to said sequence.

The present invention also includes fragments of the binding agents or of the present invention or of derivatives thereof which still have the binding activity described. Preferred fragments are at least ten amino acids long, but they may be longer (e.g. up to 50 or up to 100 amino acids long).

The binding agents of the present invention are believed to be useful in the treatment or prophylaxis of several human diseases including arthritis, lupus erythematosus, Mashimotos thyroiditis, multiple sclerosis,diabetes, uveitis, dermatitis, psoriasis, urticaria, nephrotic syndrome, glomerulonephritis, inflammatory bowel disease, ulcerative colitis, Crohn's disease, Sjogren's syndrome, allergies, asthma, rhinitis, eczema, GVH, COPD, insulitis, bronchitis (particularly chronic bronchitis) or diabetes (particularly Type 1 diabetes). They may also be useful in studying the interactions between CD23 and various ligands e.g. between CD23 and CD21, between CD23 and CD11b, between CD23 and CD11c, between CD23 and the aforesaid 70 to 85 KDa endothelial cell protein (which may be an 80 or 85 KDa endothelial cell protein) or between CD23 and a 115 KDa endothelial protein (which is believed to be related to the 70 to 85 KDa endothelial protein). One or more of the above interactions are believed to occur, *in vivo*. Antibodies or other binding agents which are capabl of blocking these

interactions are particularly preferred since it is believed that they may be especially suitable for reducing or alleviating cytokine mediated inflammatory effects. They may also be useful against B-cell malignancies such as chronic lymphocytic leukaemia, and hairy cell leukaemia.

Binding agents of the present invention are particularly applicable for use in the treatment or prophylaxis of rheumatoid arthritis. Without being bound by theory, the following possible explanations are put forward:-

In the rheumatoid arthritis inflamed synovium, macrophages express both CD23 and the β_2 integrins CD11b and CD11c, allowing possible homotypic interactions to take place in this tissue. In addition, diffusion of soluble CD23 molecules through the synovium and their binding to the integrin ligands is also possible. Therefore, CD23-CD11b/CD11c interactions involving a positive activation loop could exist *in vivo*. If present in rheumatoid arthritis patients, it may explain some of the pathogenic mechanisms of disease exacerbation and chronicity, and would support the hypothesis that once localised to the joints, macrophages themselves can maintain and exacerbate inflammation via a pathway involving CD23 molecules, β_2-integrins CD11b and CD11c, as well as pro-inflammatory cytokines TNF-α, IL-1β and IL-6.

The present inventors have found that CD23 binding to CD11b and CD11c is blocked by Factor X since Factor X binds to CD11b and CD11c. Thus the present invention includes the use of Factor X or of a fragment thereof to block CD23 binding to CD11b and/or CD11c.

An alternative mechanism of action of anti CD23 therapy could involve the blocking of an IgE immune response.

In previously published work, it has been shown that *in vivo* treatment of rats with anti-CD23 antibody resulted in antigen-specific inhibition of IgE production, probably by blocking the CD23-CD21 interactions necessary for complete differentiation of IgE-committed B cells (Flores-Romo *et al.*, *Science* **261**, 1038-1041 (1993)).

The present invention also includes binding agents to CD21 which block such a response (e.g. the Epstein Barr virus or a part thereof).

Structurally, the CD21 protein is composed of an extracellular domain of 15 (Moore *et al*, Molecular cloning of the cDNA encoding the Epstein Barr Virus C3d receptor (complement receptor type 2) of human B lymphocyte, *Proc Natl Acad Sci USA* **84**: 9194 (1987)) or 16 (Weis *et al*, Structure of the human B lymphocyte receptor for C3d and the Epstein Barr Virus and relatedness to other members of the family of C3/C4 binding proteins, *J Exp Med* **167**: 1047 (1988)) repetitive units of 60 to 75 amino acids, named short consensus repeats (SCRs), followed by a transmembrane domain (24 amino acids) and an intracytoplasmic region of 34 amino acids. Using CD21 mutants bearing deletions of extracytoplasmic SCRs (Carel *et al*, Structural requirements for C3d,g/Epstein Barr Virus receptor (CR2/CD21) ligand binding, internalization, and viral infection *J Biol Chem* **265**: 12293 (1990)), the present inventors have recently found that CD23 binds to SCRs 5-8 and 1-2 on CD21. The binding of CD23 to SCRs 5-8 is a lectin-like interaction, involving carbohydrates on Asn 295 and 370. In contrast, CD23 binding to SCRs 1-2 is a protein-protein interaction (Aubry *et al*, CD23 interacts with a new functional extracytoplasmic domain involving N-linked oligosaccharides on CD21, *J Immunol* **152**: 5806 (1994)). The present inventors have now tested the effect of the other ligands of CD21 (EBV, C3d,g and IFN-α) on CD23 binding to CD21 and on th regulation of Ig production in the presence of IL-4. Only EBV particles and an EBV-d rived peptide were able to inhibit CD23 binding to CD21. Moreover, th

EBV-peptide selectively decreased IgE and IgG4 production and increased IgM production. These data indicate that CD23 binding to the EBV-binding site on CD21 selectively regulates human Ig production in the presence of IL-4.

The present invention therefore includes within its scope the use of Epstein Barr Virus or of a part thereof to block the binding of CD23 to CD21. A preferred part of the Epstein Barr Virus is the gp350/gp220 glycoprotein or a fragment thereof. Alternatively, an unglycosylated form of this glycoprotein or of a fragment thereof may be used.

Again without being bound by theory, it is believed that the present invention allows effective treatments to be achieved by suppressing the *de novo* synthesis of pro-inflammatory cytokines.

This contrasts with previous uses of antibodies simply to directly neutralise the cytokine molecules already present in inflamed tissues.

It should also be noted that there are speculative publications in the art listing large numbers of antibodies as well as large numbers of possible diseases which the antibodies are said to be useful in treating, but not providing any sound evidence or data for most of the possible combinations. One such publication is WO93/02108 which is primarily directed to the production of particular chimaeric antibodies.

The present invention is clearly distinguished from such publications by providing binding agents to particular molecules which are clearly indicated to be of utility in the treatment or prophylaxis of certain diseases in view of the data and explanations provided herein.

Binding agents of this invention are also of particular use in the treatment or prophylaxis of allergic diseases, including non-IgE mediated diseases. They may be used in the treatment and propylaxis of ulcerative colitis. They may also be used in the treatment and prophylaxis of Crohn's disease.

The binding agents of the present invention may be used alone or in combination with immunosuppressive agents such as steroids, cyclosporin, or antibodies such as an anti-lymphocyte antibody or more preferably with a tolerance-inducing, anti-autoimmune or anti-inflammatory agent such as a CD4+T cell inhibiting agent e.g. an anti-CD4 antibody (preferably a blocking or non-depleting antibody), an anti-CD8 antibody, a TNF antagonist e.g. an anti-TNF antibody or TNF inhibitor e.g. soluble TNF receptor, or agents such as NSAIDs.

The binding agent will usually be supplied as part of a sterile, pharmaceutically acceptable composition. This pharmaceutical composition may be in any suitable form, depending upon the desired method of administering it to a patient. It may be provided in unit dosage form and may be provided as part of a kit. Such a kit would normally (although not necessarily) include instructions for use.

Binding agent administrations are generally given parenterally, for example intravenously, intramuscularly or sub-cutaneously. The binding agents are generally given by injection or by infusion. For this purpose a binding agent is formulated in a pharmaceutical composition containing a pharmaceutically acceptable carrier or diluent. Any appropriate carrier or diluent may be used, for example isotonic saline solution. Stabilizers may be added such as a metal chelator to avoid copper-induced cleavage. A suitable chelator would be EDTA, DTPA or sodium citrate.

They may be given orally or nasally by means of a spray, especially for treatment of respiratory disorders.

They may be formulated as creams or ointments, especially for use in treating skin disorders.

They may be formulated as drops, or the like, for administration to the eye, for use in treating disorders such as vernal conjunctivitis.

For injectable solutions, excipients which may be used include, for example, water, alcohols, polyols, glycerine, and vegetable oils.

The pharmaceutical compositions may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts (substances of the present invention may themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain other therapeutically active agents.

Suitable dosages of the substance of the present invention will vary, depending upon factors such as the disease or disorder to be treated, the route of administration and the age and weight of the individual to be treated. Without being bound by any particular dosages, it is believed that for instance for parenteral administration, a daily dosage of from 0.01 to 50 mg/kg of a binding agent of the present invention (usually present as part of a pharmaceutical composition as indicated above) may be suitable for treating a typical adult. More suitably the dose might be 0.05 to 10 mg/kg, such as 0.1 to 2 mg/kg.

This dosage may be repeated as often as appropriate. Typically administration may be 1 to 7 times a weeks. If side effects develop the amount and/or frequency of the dosage can be reduced.

A typical unit dose for incorporation into a pharmaceutical composition would thus be at least 1 mg of binding agent, suitably 1 to 1000 mg.

The present invention includes within its scope an assay for determining whether or not a particular agent which binds to CD21, CD11b, CD11c or to a 70-85 or 115 KDa protein expressed on endothelial cells may be useful in the treatment of an inflammatory, autoimmune or allergic disease comprising: determining whether or not the agent is capable of blocking the interaction between CD23 and CD11b, or the interaction between CD23 and CD11c, or the interaction between CD23 and CD21, or the interaction between CD23 and a 70-85 KDa or 115 KDa protein expressed on endothelial cells.

This assay can be used for screening compounds or molecules by using cell lines expressing the appropriate molecules. Preferably CD11b is used in these assays as CD11b/CD18 and CD11c is used as CD11c/CD18. CD11b/CD18 and CD11c/CD18 can be co-expressed on cell surface.

Any appropriate assay technique can be used, e.g. protein-non protein assays (e.g. assaying the interaction of proteins with chemicals or sugars), protein-protein assays or protein-cell assays.

The present invention will now be described by way of example only with reference to the accompanying drawings; wherein:

FIGURE 1a illustrates CD23-liposomes binding to CD14 positive blood mononuclear cells;

FIGURE 1b illustrates various CD23 affinity purified proteins on SDS-PAGE gels;

FIGURE 2 illustrates the percentage inhibition of CD23-liposome binding to activated blood monocytes obtained using certain monoclonal antibodies;

FIGURE 3 illustrates the binding of CD23 liposomes to various transfected cells;

FIGURE 4 illustrates the effect of various substances on CD23-CD11b and CD23-CD11c interaction;

FIGURE 5 illustrates the effects on nitrite production and oxidative burst in monocytes caused by CD23 binding to CD11b and CD11c; and

FIGURE 6 illustrates that the binding of recombinant CD23 to CD11b and CD11c specifically increases cytokine production by monocytes.

FIGURE 7a illustrates the inhibition of CD23-liposome binding to RPMI 8226 cells by various CD21 ligands.

FIGURE 7b illustrates the inhibition of IL-4 induced IgE and IgG4 production by an EBV peptide binding to CD21.

FIGURE 7c illustrates the modulation of immunoglobulin production by an EBV peptide binding to CD21.

FIGURE 7d illustrates the absence of inhibition of IgE production with a C3 peptide binding to CD21.

FIGURE 8 illustrates the inhibition of the binding of CD23 liposomes to certain endothelial cells due to the presence of an anti-CD23 MAb.

EXAMPLES

(In some of the following examples the terms "ip", "id" and "n" are used. These mean "intraperitoneal", "intradermal" and "number of animals" respectively.)

EXAMPLES 1-6

Interaction between CD23 and CD11b and between CD23 and CD11c

Examples 1 to 6 and the accompanying Figures (see later) illustrate the interaction of CD23 with CD11b and/or CD11c.

In these Examples, full-length recombinant CD23 incorporated into fluorescent liposomes was shown to bind to COS cells transfected with cDNA encoding either CD11b/CD18 or CD11c/CD18 but not with transfectants expressing CD11a/CD18. The interaction between CD23-liposomes and CD11b/CD18 or CD11c/CD18-transfected COS cells was specifically inhibited by anti-CD11b or anti-CD11c, respectively, and by anti-CD23 monoclonal antibodies. The functional significance of this ligand pairing was demonstrated by triggering CD11b and CD11c on monocytes with either recombinant CD23 or anti-CD11b and anti-CD11c monoclonal antibodies to cause a marked increase in nitrite (NO_2^-), oxidative products (H_2O_2) and proinflammatory cytokines (IL-1β, IL-6 and TNFα). These CD23-mediated activities were decreased by Fab fragments of monoclonal

antibodies to CD11b, CD11c and CD23. These results demonstrate that the surface adhesion molecules CD11b and CD11c are receptors for CD23 and that this novel ligand pairing regulates important activities of monocytes.

The following discussion explains briefly the experimental design and the rationale behind Examples 1 to 6 (which follow):-

Total blood mononuclear cells were incubated with recombinant full-length CD23 incorporated into fluorescent liposomes and analysed by flow cytometry (Pochon, S. *et al. J. Exp. Med.* **176**, 389-398 (1992)). A fraction bound CD23-liposomes (Example 1, Fig. 1a) which was then shown by double staining to consist of CD14-positive cells (i.e. monocytes). To confirm that monocytes were able to bind CD23-liposomes, blood mononuclear cells were FACS-sorted into CD14-positive and CD14-negative populations (Example 1, Fig. 1a). CD23-liposomes were shown to bind only to the CD14-positive population (Example 1, Fig. 1a). Since monocytes were found to express neither membrane IgE nor CD21 (not shown), the known ligands for CD23, it was investigated whether monocytes express a different receptor for CD23. Monocytes were lysed and cell extracts purified over an affinity column coupled with recombinant soluble CD23. SDS-PAGE and silver staining analysis of the eluted material revealed bands of around 80 and 160 kDa MW (Example 1, Fig. 1b). Antibodies identifying antigens within this range of MW and reported to be expressed on monocytes were tested by FACS for their capacity to inhibit CD23-liposome binding to monocytes (Example 2, Fig. 2). Anti-CD11b and anti-CD11c monoclonal antibodies both inhibited CD23-liposome binding to monocytes, with varying degrees of potency (Example 2, Fig. 2). Anti-CD13, anti-CD49d, anti-CD21 (not expressed on monocytes) and anti-CD11a (the third member of the β2 integrin family of adhesion molecules) had no significant effect (Example 2, Fig. 2). Antibodies against MHC Class I, Class II, CD14 and CD45, all of which highly expressed on monocytes, were also tested for their effect on CD23-

liposome binding. None however had any effect (not shown). Anti-CD18 monoclonal antibody gave a partial inhibition of CD23 binding. This could be due either to steric hindrance or to the induction of a conformational change in the CD11b and CD11c molecules upon anti-CD18 Mab binding. The monocyte-derived proteins eluted from the CD23-affinity column were immunoreactive with anti-CD11c (Example 1, Fig. 1b) and anti-CD11c/CD18 antibodies (not shown).

To confirm that the α chain of CD11b/CD18 and CD11c/CD11b were receptors for CD23, full-length cDNAs encoding CD11b and CD11c were transiently co-transfected with CD18 cDNA into COS cells. Transfectants expressing CD11b/CD18 and CD11c/CD18 were both shown to bind CD23-liposomes, in contrast to transfectants expressing CD11a/CD18 (Example 3, Fig. 3). This might be explained by the higher degree of homology between CD11b and CD11c when compared to their homology to CD11a. The specificity of the interaction was demonstrated by inhibiting CD23-liposome binding using anti-CD11b, anti-CD11c and anti-CD23 monoclonal antibodies. The same results were obtained using BHK cells expressing CD11b/CD18 and CD11c/CD18 (not shown). As further proof of the specificity of the CD23 interaction, activated blood monocytes from a Leukocyte Adhesion Deficiency patient, lacking β2 integrin expression due a mutation in the gene encoding the β subunit were unable to bind CD23-liposomes (not shown). Together, these data demonstrate that CD23 interacts with CD11b and CD11c on normal human monocytes and on transfectants.

CD11b and CD11c are adhesion molecules that participate in many cell-cell and cell-matrix interactions. The examples show that CD11b/CD18 and CD11c/CD18 may exhibit an additional adhesive function by virtue of their ability to bind CD23. CD23 seems to identify an epitope close or identical to factor X as observed by the capacity of factor X t inhibit in a dose dependent manner CD23-liposom binding (Example 4, Fig. 4) without affecting surface expression of CD11b or CD11c on

monocytes (not shown). None of the other ligands tested had any effect. CD23 may be acting as a C-type lectin in its interaction with CD11b and CD11c. EDTA decreases CD23 binding to monocytes (Example 4, Fig. 4) by chelation of Ca^{2+} which is necessary to CD23 binding and/or by chelation of the divalent cations which are necessary for the binding of ligands to CD11b and CD11c (Altieri, D.C. *J. Immunol.* **147**, 1891-1898 (1991)). CD23-CD11b/CD11c interaction seems to involve sugars, but not sialic acid, as observed by the capacity of tunicamycin, but not neuraminidase, to decrease CD23 binding to monocytes. CD23 bears extracellularly a triplet of amino acids (Asp, Gly, Arg) (Kikutani, H. *et al. Cell* **47**, 867-885 (1986)), which in the reverse orientation is a common recognition site for the integrin receptors. Therefore, the effect of a polyclonal antibody directed against this tripeptide was tested for its capacity to inhibit CD23 binding to monocytes. No inhibition was observed, confirming the absence of inhibition obtained with fibrinogen (Example 4. Fig. 4). IgE which is binding in the lectin domain of CD23, partially inhibits CD23 binding to monocytes (Example 4, Fig. 4). Those results indicate that CD23 would seem to be acting as a C-type lectin recognising partly sugar and protein structures, reminiscent of what has been observed for CD23 interaction with CD21 (Aubry, J-P. *et al. J. Immunol.* **152**, 5806-5813 (1994)).

To evaluate the functional significance of the interaction of CD23 with CD11b or CD11c, we investigated whether CD23-CD11b/CD11c interaction could trigger monocytes to release proinflammatory mediators such as nitric oxide, H_2O_2 and cytokines. Triggering of adherence-activated normal monocytes using recombinant soluble CD23, anti-CD11b or anti-CD11c antibodies increased the generation of NO_2^- indicating the activation of the NO pathway (Moncada, S., Palmer, R.M.J. & Higgs, E.A. *Pharmacol. Rev.* **43**, 109-144 (1991)). The effect of CD23 on nitrite production was inhibited by Fab fragments of anti-CD23 monoclonal antibodies and by nitroarginine, a specific inhibitor of the NO synthase

pathway (Example 5, Fig. 5a). The oxidative burst was also shown to be regulated through CD11b and CD11c since recombinant soluble CD23, anti-CD11b and anti-CD11c monoclonal antibodies all caused oxidation of hydroethidine to ethidium bromide in monocytes (Example 5, Fig. 5b). This confirms and extends the finding that anti-CD11b monoclonal antibodies induce an oxidative burst in monocytes (Trezzini, C., Schüepp, B., Maly, F.E. & Jungi, T.W. *Brit. J. Haematol.* **77**, 16-24 (1991)). CD23 binding to CD11b and CD11c was associated with an early specific Ca^{2+} flux in blood monocytes (not shown).

Since activated macrophages are an important source of proinflammatory cytokines, we evaluated the effect of recombinant soluble CD23 and of anti-CD11b and anti-CD11c monoclonal antibodies on the production of such cytokines by monocytes. Recombinant soluble CD23, anti-CD11b and anti-CD11c monoclonal antibodies were potent stimulators of IL-1β, IL6 and TNFα. Again, the specificity of this induction was demonstrated by using Fab fragments of anti-CD11b, anti-CD11c and anti-CD23 monoclonal antibodies (Example 6, Fig. 6). Interestingly, IL-1 and TNFα were potent inducers of CD23-liposome binding to monocytes (not shown), suggesting a potential cytokine autocrine loop through CD11b and CD11c stimulation and regulation.

EXAMPLE 1

a) CD23-liposomes bind to CD14-positive blood mononuclear cells (See Fig. 1a).

Blood mononuclear cells were stained with anti-CD14 monoclonal antibody (Becton Dickinson, Erembodegem, Belgium) followed by sheep FITC-conjugated $F(ab')_2$ antibodies to mouse IgG and IgM (Bioart, Meudon, France), both diluted in PBS, 0.5% BSA and 0.05% sodium azide prior to FACS-sorting (FACStar Plus, Becton Dickinson) into CD14-positive and CD14-negative cell populations. Separated cells were then stained with CD23-liposomes or control (glycophorin A)-

liposomes diluted in 0.5% BSA, 0.1% sodium azide, 2 mM $CaCl_2$, 140 mM NaCl, 20 mM Hepes, pH 7 and incubated for 2 h at 4°C (Pochon, S. *et al.*, *J. Exp. Med.* **176** 389-398 (1992)). After washes, cells (5,000 events/condition) were analysed by FACS.

b) Apparent molecular weight of CD23-affinity purified blood monocyte proteins and immunoreactivity with an anti-CD11c monoclonal antibody (See Fig. 1b).

Lysates of blood monocytes were affinity purified on a CD23-column, eluted proteins separated on SDS-PAGE gels and transferred onto nitrocellulose. Mr markers are shown on the left. The gel was silver stained (left lane). Filters were incubated with either an isotype matched antibody (middle lane) or with an anti-CD11c monoclonal antibody (BU-15, right lane), then with horseradish peroxidase-conjugated goat anti-mouse antibody (Kpl; Gaithersburg, Massachusetts).

EXAMPLE 2

Anti-CD11b and anti-CD11c monoclonal antibodies decrease CD23-liposome binding to activated blood monocytes (See Fig. 2).

Monocytes were enriched from mononuclear cells by Ficoll and overnight adherence to plastic in RPMI 1640 (Seromed, Berlin, Germany) supplemented with 2 mM glutamine and 10% heat-inactivated FCS (Flow Laboratories, Irvine, Scotland). Activated monocytes were then incubated with CD23-liposomes in the presence of different monoclonal antibodies (αCD) or isotype-matched controls (CTRL) (Becton Dickinson), all tested at 10 µg/ml. Anti-CD11a monoclonal antibodies 25.3 and B-B15 were obtained from Immunotech (Luminy, France) and Serotec (Oxford, UK), respectively. Anti-CD11b monoclonal antibody 44 was from Serotec, mon.gran 1 was from Janssen (Beerse, Belgium), Leu-15 was from Becton Dickinson (Erembodegem, Belgium) and (Bear-1) was from Sera-Lab Ltd (Sussex, GB). Anti-CD11c monoclonal antibody 3.9 was from Serotec, SL9 was

from Sera-Lab and BU-15 was from The Binding Site (Birmingham, UK). Anti-CD13 (SJ1D1), anti-CD18 (BL5), anti-CD23 (mAb25) and anti-CD49d (HP2.1) monoclonal antibodies were from Immunotech. Anti-CD21 monoclonal antibody BL13 was from Immunotech, OKB7 from Ortho and BU-33 was obtained from Dr. MacLennan (Birmingham University, UK), HB-5 from ATCC, OKB7 from Ortho Diagnostics System Inc (Raritan, NJ). Anti-CD14, anti-CD3, anti-CD16 and anti-CD20 monoclonal antibodies were from Becton-Dickinson. Cells were analysed by FACS and mean fluorescence intensity (MFI) measured. Data of a representative experiment are presented. MFI of cells stained with control-liposomes was 6.5 and with CD23-liposomes was 84.5. Percentage inhibition using arithmetic linear MFI values is calculated according to the following formula:

$$\% \text{ inhibition} = MFI \frac{[(CD23\text{-lipo})]-[(CD23\text{-lipo})+Mab]}{(CD23\text{-lipo})} \times 100$$

EXAMPLE 3

CD23-liposomes bind to α chains of CD11b/CD18 and CD11c/CD18 on recombinant transfectants (See Fig. 3). cDNAs coding for CD11a (Corbi, A.L., Miller, L.J., O'Connor, K., Larson, R.S. & Springer, T.A. *EMBO J.* **6**, 4023-4028 (1987)) was recloned in pCDNA1 (Invitrogen, San Diego, CA). cDNA for CD11b (Corbi, A.L., Kishimoto, T.K., Miller, L.J. & Springer, T.A. *J. Biol. Chem.* **263**, 12403-12411 (1988)) and CD18 (Kishimoto, T.K., O'Connor, K., Lee, A., Roberts, T.M. & Springer, T.A. *Cell* **48**, 681-690 (1987)) were recloned in pCDM8 (Seed, B., *Nature* **329** 840-842 (1987)). 20 µg aliquots of DNA were transfected in COS-7 cells (ATCC) by electroporation (260 V, 960 µFD) using a Gene Pulser device (Bio-Rad, Richmond, CA) and 0.4 cm cuvettes in 20 mM Hepes pH 7.4, 150 mM NaCl. Co-transfections of CD11a; b or c with CD18 were performed in order to get expression of the β2 integrins at the cell surface. Controls were done with single

chain transfections. 48 h after transfection, COS cells were stained with anti-CD11a, anti-CD11b and anti-CD11c monoclonal antibodies or isotype-matched monoclonal antibodies (control) followed by FITC-labelled sheep anti-mouse antibody. Between 10 to 15% of the cells were shown to express CD11a, b, c or CD18 by staining with the respective monoclonal antibodies. Prior to staining with CD23-liposomes, CD18-positive transfected COS cells were then FACS-sorted in order to increase the percentage of cells expressing β2 integrins. CD11a/CD18, CD11b/CD18 and CD11c/CD18 transfectants were then incubated with CD23-liposomes (trace 2) or control (glycophorin A)-liposomes (trace 1). The specificity of CD23 interaction with CD11b and CD11c was demonstrated by inhibition of CD23-liposome binding to CD11b/CD18 and CD11c/CD18 transfectants using anti-CD11b (trace 4); anti-CD23 (trace 5) and anti-CD11c (trace 6) monoclonal antibodies, respectively. No binding of CD23-liposomes was observed on CD11a/CD18 transfectants and no effect of anti-CD11a monoclonal antibody was found (trace 3).

EXAMPLE 4

Structural characterisation of CD23-CD11b, CD11c interaction (See Fig. 4).

(a) Involvement of sugars and divalent cations.

Purified activated blood monocytes were treated or not with tunicamycin (10 μg/ml) for 48 h or with neuraminidase (0.1 U/ml; both from Boehringer Mannheim, Mannhein, Germany) for 45 min. Cells were then incubated with CD23-liposomes or control-liposomes in the absence or presence of EDTA (5 mM; top left panel), Ca^{2+} or Mn^{2+} (1 to 10 mM; top right panel).

(b) Factor X does inhibit CD23 binding to monocytes. Purified activated blood monocytes were incubated with CD23-liposomes in absence or presence of factor X (0.1 to 10 U/ml; Sigma) (bottom left panel), fibrinogen (50 μg/ml; Sigma), purified recombinant ICAM-1 (produced in our laboratory), LPS (1 μg/ml; Sigma), human

serum opsonised-zymosan (1 mg/ml; Sigma), IgE (50 µg/ml; The Binding Site, Birmingham) or polyclonal antibody to RGD peptide (1/500, ATCC) (bottom right panel). Cells were analysed by FACS and MFI measured. Percentage inhibition was calculated as for Example 2.

EXAMPLE 5

Recombinant CD23 by binding to CD11b and CD11c specifically increases a, the nitrite product and b, the oxidative burst by monocytes.

Monocytes were incubated a, for 4 days at 37°C or b, overnight in the absence or presence of recombinant soluble CD23 (Graber P. *et al.*, *J. Immunol. Methods* **149** 215-226 (1992)) (50 ng/ml), anti-CD11a (clone 25.3), anti-CD11b (clone 44), anti-CD11c (clone BU-15) monoclonal antibodies (all at 10 µg/ml).

To assess the amount of NO produced (which is shown in Fig. 5a), the culture supernatants were assayed for the stable end products of NO, NO_2^- and NO_3^- according to Green *et al.*, *Annu. Rev. Immunol.* **2** 199-218 (1984). The specificity of CD23-mediated increase of NO_2^- production was demonstrated by inhibition of NO_2^- production by Fab fragments of anti-CD23 monoclonal antibodies (mab25) (tested at 10 µg/ml) and by inhibition with nitroarginine (N-Arg at 1 mM) (Sigma).

Activated monocytes were incubated with hydroethidine (Molecular probes, Eugene, OR) (0.3 µg/ml) for 30 min at 37°C (Rothe G. *et al.*, *J. Leukoc. Biol.* **47** 440-448 (1990)) and analysed by FACS. Percentage increase in red fluorescence of stimulated monocytes is shown in comparison to untreated monocytes (See Fig. 5b). Monocytes which had undergone an oxidative burst shown an increase of red fluorescence signals compared to untreated monocytes reflecting oxidation of hydroethidine to ethidium bromide (Lacal P.M. *et al.*, *Biochem. J.* **268** 707-712 (1990)). MFI values of monocytes alone were 159+/-10. Mean+/-SD values of 6 experiments are presented. Con A, which is known to induce a respiratory burst in

monocytes, was used as a positive control. The specificity of the CD23 interaction with CD11b and CD11c was demonstrated by inhibition of CD23-mediated increase of H_2O_2 production by Fab fragments of anti-CD11b (clone 44), anti-CD11c (clone BU-15) and anti-CD23 (mAb25) monoclonal antibodies (tested at 10 µg/ml).

EXAMPLE 6

Binding of recombinant CD23 to CD11b and CD11c specifically increases cytokine production by monocytes (See Fig. 6).

Monocytes were incubated overnight at 37°C in the absence or presence of recombinant soluble CD23 (Graber P. *et al., J. Immunol. Methods* **149** 215-226 (1992)) (50 ng/ml), anti-CD11a (clone 25.3), anti-CD11b (clone 44), anti-CD11c (clone BU-15), anti-CD23 (mAb 25 - this antibody is available from Immunotech. It is discussed in published European Patent Application EP-A-0269728) monoclonal antibodies, Con A (Sigma) (all at 10 µg/ml), LPS (1 ng/ml) (Sigma) or PMA (5 ng/ml) (Calbiochem, La Jolla, CA). Cytokines were measured in the culture supernatant by specific ELISA. The ELISA's limit of sensitivity is 0.05 ng/ml for IL-1β (Ferrua *et al., J. Immunol. Methods* **114** 41-48 (1988)) 0.01 ng/ml for TNFα (Medgenix, Biotechnie, Rungis, F) and <0.01 ng/ml for IL-6 (Manie *et al., Eur. Cytokine Netw.* **4** 51-56 (1993)). The specificity of CD23 interaction with CD11b and CD11c was demonstrated by inhibition of CD23-mediated increase of cytokine production by Fab fragments of anti-CD11b (clone 4), anti-CD11c (clone BU-15) and anti-CD23 (mAb25) monoclonal antibodies (tested at 10 µg/ml). Mean+/-SD values of 4 experiments are presented.

EXAMPLE 7

The following materials and methods were used in this example:

Cell Lines

Tonsil or blood mononuclear cells were separated into T and B cell subpopulations by rosetting with sheep red blood cells.

The B cell line RPMI 8226 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 complete medium.

Peptides and CD21 ligands

Two peptides, from gp350 of EBV and C3, known to bind to CD21 (Servis *et al*, C3 synthetic peptides support growth of human CR2-positive lymphoblastoid B cells, *J Immunol* **142**: 2207 (1989)) were synthesized. PepEBV (TGEDPGFFNVEIC-NH2) was produced on an ABI 431A synthesizer using FastMoc chemistry and PepC3 (GKQLYNVEATSYAC-NH2) was obtained from Neosystem (Strasbourg, France). Aggregated C3d,g was prepared as described previously (Carel *et al* (1990) *supra*). Sucrose gradient purified EBV was obtained from Advanced Biotechnologies (Columbia, ML) and IFN-α was obtained from Sigma (St Louis, MO).

Liposome preparation

CD23-liposomes were made as previously described (Pochon *et al*, Demonstration of a second ligand for the low affinity receptor for immunoglobulin E (CD23) using recombinant CD23 reconstituted into fluorescent liposomes, *J Exp Med* **176**: 389 (1992)) using 10 μmoles of the synthetic phospholipids POPC (Avanti Polarlipids Inc. Alabaster, AL) mixed with 50 nmoles of fluorescent dye DiO18 (Molecular Probes, Eugene, OR) and then dialyzed against HEPES buffer together with purified recombinant CD23 or with glycophorin A (0.2 μmoles each) referred to as control protein.

Fl w cytometry

Liposome binding assay

Cells (10^5) were resuspended in 50 µl of the liposome suspension, diluted 10 times in 0.5% BSA, 0.1% NaN_3, 2 mM $CaCl_2$, 140 mM NaCl, 20 mM Hepes, pH 7.0 and incubated for 2 h at 4°C.

Cells were washed twice before analysis on a FACStar plus (Becton Dickinson, Erembodeggen, Belgium).

Competition of CD23-liposomes with EBV, EBV peptide, IFN-α, C3 peptide and C3d,g

RPMI 8226 cells were co-incubated with glycophorin-liposomes or CD23-liposomes and EBV ($1x10^5$ to $1x10^9$ particles/ml), EBV peptide and C3 peptide (50 nM to 50 µM), aggregated C3d,g (4 ng/ml to 1 µg/ml) and IFN-α (1000 U/ml) for 2 h at 4°C. Cells were analyzed as described above.

IL-4-induced Ig production assays

Cells were incubated at 10^6/ml for 14 days in Iscove's medium enriched with transferrin, bovine insulin, oleic acid, linoleic acid, palmitic acid, BSA (all from Sigma) and 10% FCS (Flow Laboratories, Irvine, Scotland) as described by Claassen *et al* (A cell culture system that enhances mononuclear cells IgE synthesis induced by recombinant interleukin-4, *J Immunol Methods* **126**: 213 (1990)). Assays were performed using total PBMNC with IL-4 alone (200 U/ml) or IL-4 plus anti-CD40 (1 µg/ml) (Serotec Ltd. Oxford, UK), or using purified tonsillar B cells with IL-4 and anti-CD40. IgE, G, A and M were quantified by specific ELISA as previously described (Bonnefoy *et al*, Inhibition of human interleukin-4-induced IgE synthesis by a subset of anti-CD23/Fc epsilon RII monoclonal antibodies, *Eur J Immunol* **20**: 139 (1990)). IgG4 was measured by ELISA as follows. A mouse anti human IgG4 antibody (Southern Biotechnology, Birmingham) diluted at 10 µg/ml in bicarbonate buffer, pH 9.6 was coated overnight in 96 well plates (100 µl/well). Saturation was then performed with PBS

plus 1% BSA (200 µl/well) for 2 h at RT. Samples to be tested were diluted in PBS plus 0.5% BSA and 0.1% Tween (100 µl/well) and incubated overnight at 4°C. After washes with PBS plus Tween, a peroxidate-labelled sheep anti human IgG4 antibody (Vital products, St Louis, MO) diluted 1/5000 in PBS/BSA plus Tween was added for 1 h at RT. After washes with PBS plus Tween, o-phenylene diamine (Sigma) is added and the colorimetric reaction was stopped with 2M H_2SO_4. Plates were finally read at 492 nm.

The results obtained are discussed below:

Human CD21 has been previously described to be a receptor for the C3d,g and iC3b proteins of the complement system (Weis *et al*, Identification of a 145,000 Mr membrane protein as the C3d receptor (CR2) of human B lymphocytes *Proc Natl Acad Sci USA* **81**: 881 (1984)), for the gp350/220 envelope glycoprotein of EBV (Nemerow *et al*, Identification of gp 350 as the viral glycoprotein mediating attachment of Epstein Barr Virus (EBV) to the EBV/C3d receptor of B cells : sequence homology of gp350 and C3 complement fragment C3d, *J Virol* **61**: 1416 (1987); Tanner *et al*, Epstein Barr Virus gp 350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping and endocytosis, *Cell* **50**: 203 (1987)) and for IFN-α (Delcayre *et al*, Epstein Barr Virus/complement C3d receptor is an interferon α receptor, *EMBO J* **10**: 919 (1991)). We have therefore tested all these CD21 ligands for their ability to inhibit CD23-liposome binding to the CD21-expressing cells, RPMI 8226 cells (Pochon *et al* (1992) *supra*). Intact particles of EBV were able to inhibit CD23 binding to CD21 in a dose dependent manner. This is shown in Figure 7a, which illustrates the inhibition of CD23-liposome binding to RPMI 8226 cells by some CD21 ligands. [RPMI 8226 cells were co-incubated for 2 h with CD23-liposomes or glycophorin-liposomes and various concentrations of EBV (particles/ml), PepEBV and PepC3 (µM). Percentage of inhibiti n is calculated as follows:

$$\frac{(\text{MFI CD23-L}) - (\text{MFI CD23-L + ligands})}{(\text{MFI CD23-L})} \times 100$$

The MFI of glycophorin-liposomes was subtracted from the MFI of CD23-liposomes. Inset= FACS profiles of RPMI 8226 cells stained with glycophorin-liposomes (traces 1) or CD23-liposomes (traces 3) alone or in presence of EBV particles (top), PepEBV (medium) and PepC3 (bottom) (traces 2). Results are taken from a representative experiment.]

Of the other CD21 ligands tested for inhibition of CD23 liposome binding, only EBV decreased the binding of CD23. A complete inhibition of CD23 binding was observed with EBV intact particles, although EBV is reported to bind to SCR 2 of CD21 and not to SCRs 5-8 where CD23 binds to sugars in this latter region (Aubry *et al* (1994) *supra*). This complete inhibition of CD23 binding could be due to the size of the virus particles or to the fact that EBV may modify the conformation of the CD21 molecule. In order to exclude that inhibition was due to steric hindrance by the virus particles, we then tested the effect of a peptide of gp350/220, known to bind to CD21 (Servis *et al* (1989) *supra*). This EBV peptide was able to inhibit CD23 binding to CD21 in a dose dependent manner, with a maximum of 55% inhibition (Fig 7a). These experiments suggest that the EBV peptide binding is close to the CD23 binding site and partially blocks CD23 binding. These data confirm our previous finding that CD23 does bind to CD21 (Aubry *et al*, CD21 is a ligand for CD23 and regulates IgE production, *Nature* 358: 505 (1992)) and extend them by showing that SCR2 is probably a region interacting with CD23. In contrast, a C3 peptide corresponding to the CD21 binding site on C3d (Servis *et al* (1989) *supra*) (Fig 7a), aggregated C3d,g and IFN-α (data not shown) were unable to inhibit the binding of CD23 to RPMI 8226 cells. This suggests that SCRs 1 and 3-4, where C3 and IFN-α bind respectively, may not be involved in CD23 binding.

The EBV binding to CD21 does not require glycosylation of SCR2 of CD21 (Moore *et al*, Inhibition of Epstein Barr Virus infection *in vitro* and *in vivo* by soluble CR2 (CD21) containing two short consensus repeats, *J Virol* **67**: 3559 (1991)). Likewise, CD23 binding to SCR2 region is independent of sugars (Aubry *et al* (1994) *supra*). This is in line with our observation that a non-glycosylated synthetic peptide is able to decrease CD23 binding to CD21. Therefore, CD23 binds to a binding site in SCR2 on CD21 that is close or identical to the EBV binding site which differs from the binding sites previously described for C3d,g and IFN-α.

CD23 was previously shown to positively regulate IgE production by binding to CD21 on B cells (Aubry *et al* (1992) *supra*). Based on the observation that an EBV peptide blocked CD23 binding to CD21, we investigated the effect of this EBV peptide on IgE production. The EBV peptide was able to inhibit IL-4-induced IgE production in a dose-dependent manner. This is shown in Figure 7b, which illustrates the inhibition of IL-4-induced IgE and IgG4 production by an EBV peptide binding to CD21. [PBL or purified tonsillar B cells (10^6/ml) were incubated for 14 days with 200 U/ml of IL-4 alone or in the presence of anti-CD40 antibody (1 µg/ml) and increasing concentrations of EBV peptide. IgE and IgG4 were measured by specific ELISA and mean values +/- SD of one representative experiment are presented (n=4)].

This effect was observed in T-cell dependent and also in T-cell independent IgE production systems (Fig 7b), in which T-cell help is replaced by anti-CD40 Ab. This confirms our previous observation (Henchoz *et al*, Stimulation of human IgE production by a subset of anti-CD21 monoclonal antibodies : requirement of a co-signal to modulate ε transcripts, **81**: 285 (1994)) that CD23-CD21 can regulate IgE production even in absence of T-cells by a homotypic B-B cell interaction, since B cells can xpress both CD23 and CD21 m lecul s. Intact EBV has been

reported to provide the permissive signal for IgE switching (Thyphronitis *et al*, IgE secretion by Epstein-Barr virus infected purified human B lymphocytes is stimulated by interleukin-4 and suppressed by interferon-γ, *Proc Natl Acad Sci USA* **86**: 5580 (1989)), like T-cells or CD40L. In contrast to the EBV particles, the EBV peptide is probably unable to crosslink membrane CD21 and is therefore unable to increase IgE production. The EBV peptide is rather inhibitory, decreasing IgE production by preventing the CD23-CD21 interaction.

Since IL-4 is known to induce IgG4 as well as IgE (Lundgren *et al*, Interleukin-4 induces synthesis of IgE and IgG4 in human B cells, *Eur J Immunol* **19**: 1311 (1989)), we investigated the effect of the EBV peptide on IL-4-induced IgG4 production. As shown in Fig 7b the EBV peptide was also able to inhibit IgG4 in a dose dependent manner. This observation suggests that the CD23-CD21 interaction also controls IgG4 production.

The effect of the EBV peptide was then investigated on the production of other Ig classes. No significant effect was found on polyclonal IgG and IgA production. This is shown in Figure 7c, which illustrates the modulation of immunoglobulin production by an EBV peptide binding to CD21. [PBL or purified tonsillar B cells (10^6/ml) were incubated for 14 days with 200 U/ml of IL-4 alone or in the presence of anti-CD40 antibody (1 µg/ml) and increasing concentrations of EBV peptide. Ig were measured by specific ELISA and mean values +/- SD of one representative experiment are presented (n=4)].

In contrast, IgM production was significantly increased in the presence of the EBV peptide, especially when whole PBL were used (Fig 7c).

Not all CD21 ligands can regulate IgE/IgG4 production. A C3 peptide binding CD21 did not inhibit IgE and IgG4 production (not shown). This is shown in Figure

7d, which illustrates the absence of inhibition of IgE production with a C3 peptide binding to CD21. [Purified tonsillar B cells (10^6/ml) were incubated for 14 days with 200 U/ml of IL-4 and anti-CD40 antibody (1 µg/ml) and increasing concentrations of C3 peptide or EBV peptide. IgE was measured by specific ELISA and mean values +/- SD of one representative experiment are presented (n=4)].

The C3 peptide did not inhibit CD23 binding (Fig 7a). These results highlight again the correlation between CD23-CD21 pairing and IgE/IgG4 production. IFN-α was not tested since it is already known that IFN-α inhibits IgE production (Pene *et al*, IgE production by normal human lymphocytes is induced by IL-4 and suppressed by α-interferon, γ-interferon, and prostaglandin E2, *Proc Natl Acad Sci USA* **85**: 8166 (1988)), although IFN-α had no effect on CD23 binding to CD21 (not shown).

In conclusion, this study therefore shows that an EBV peptide decreases CD23 binding to CD21 and selectively decreases IgE and IgG4 production by human B cells.

EXAMPLE 8 - CD23 binds to endothelial cells

An endothelial cell line (LT2, Endolethium vol 2, p 191-201, 1994) or purified human umbilical venule endothelial cells were incubated with CD23-liposomes (CD23L), or glycophorine-liposomes (L-Gly) as a control. Specificity of the binding was demonstrated by the inhibition of CD23-liposome binding by an anti-CD23 mAb (mAb25 = a-CD23). Cells were analysed by FACS and MFI measured.

The results are shown in Figure 8.

LT2 - derived proteins were purified on a CD23 - affinity column. Two bands of 115 and 76 K Da were identified.

5

CLAIMS

1. A binding agent to CD21, CD11b, CD11c, to a 70-85 KDa protein expressed on endothelial cells, or to a 115 KDa protein expressed on endothelial cells, for use in the treatment or prophylaxis of inflammatory, autoimmune or allergic diseases.

2. A binding agent according to claim 1, wherein the binding agent is an antibody, a fragment thereof, an artificial construct comprising an antibody or comprising a fragment thereof, a mimetic, or a derivative of any of these binding agents.

3. A binding agent according to claim 1 or 2 which is a humanised or chimaerised antibody.

4. A binding agent according to claim 1 which is Epstein Barr Virus, Factor X, a part of the Epstein Barr Virus (which may be in glycosylated or unglycosylated form), a fragment of Factor X, or a derivative of any of these binding agents.

5. A binding agent according to claim 4, which is the gp350/220 glycoprotein of Epstein Barr Virus, the corresponding protein in unglycosylated form, a fragment of the aforesaid glycoprotein or protein, or a derivative of any of these binding agents.

6. A binding agent according to claim 5 which is a peptide with the amino acid sequence TGEDPGFFNVEIC, a fragment thereof, or a derivative of said peptide or fragment.

7. A binding agent according to any preceding claim which blocks interaction between CD23 and ligands which bind to it *in vivo*.

8. A binding agent according to any preceding claim for use in the treatment of arthritis, lupus erythematosus, systemic lupus erythematosus, Mashimotos thyroiditis, multiple sclerosis, diabetes, uveitis, dermatitis, psoriasis, urticaria, nephrotic syndrome, glomerulonephritis, inflammatory bowel disease, ulcerative colitis, Crohn's disease, Sjogren's syndrome, allergies, asthma, eczema, GVH, COPD, bronchitis, insulitis, rhinitis or diabetes.

9. A binding agent according to any of claims 1 to 7 for use in the treatment of arthritis, allergis, ulcerative colitis or Crohn's disease.

10. A binding agent according to claims 9 for use in the treatment of rheumatoid arthritis.

11. The use of a binding agent to CD21, CD11b, CD11c, to a 70 to 85 KDa protein expressed on endothelial cells, or to a 115 KDa protein expressed on endothelial cells for the manufacture of a medicament for the treatment of arthritis, lupus erythematosus, systemic lupus erythematosus, Mashimotos thyroiditis, multiple sclerosis,diabetes, uveitis, dermatitis, psoriasis, urticaria, nephrotic syndrome, glomerulonephritis, inflammatory bowel disease, ulcerative colitis, Crohn's disease, Sjogren's syndrome, allergies, asthma, eczema, GVH, COPD, bronchitis, insulitis, rhinitis or diabetes.

12. The use of a binding agent according to claim 11 for the manufacture of a medicament for the treatment of arthritis, allergies, ulcerative colitis, or Crohn's disease.

13. The use of a binding agent according to claim 12 for the manufacture of a medicament for the treatment of rheumatoid arthritis.

14. A pharmaceutical composition comprising a binding agent according to any of claims 1 to 7 and a pharmaceutically acceptable carrier.

15. A binding agent according to claim 1 substantially as hereinbefore described.

16. A method of treating an inflammatory, autoimmune or allergic disease comprising administering a pharmaceutically effective amount of a binding agent to CD21, CD11b, CD11c, to a 70 to 85 KDa protein expressed on endothelial cells, or to a 115 KDa protein expressed on endothelial cells, to a patient.

FIG. 1a

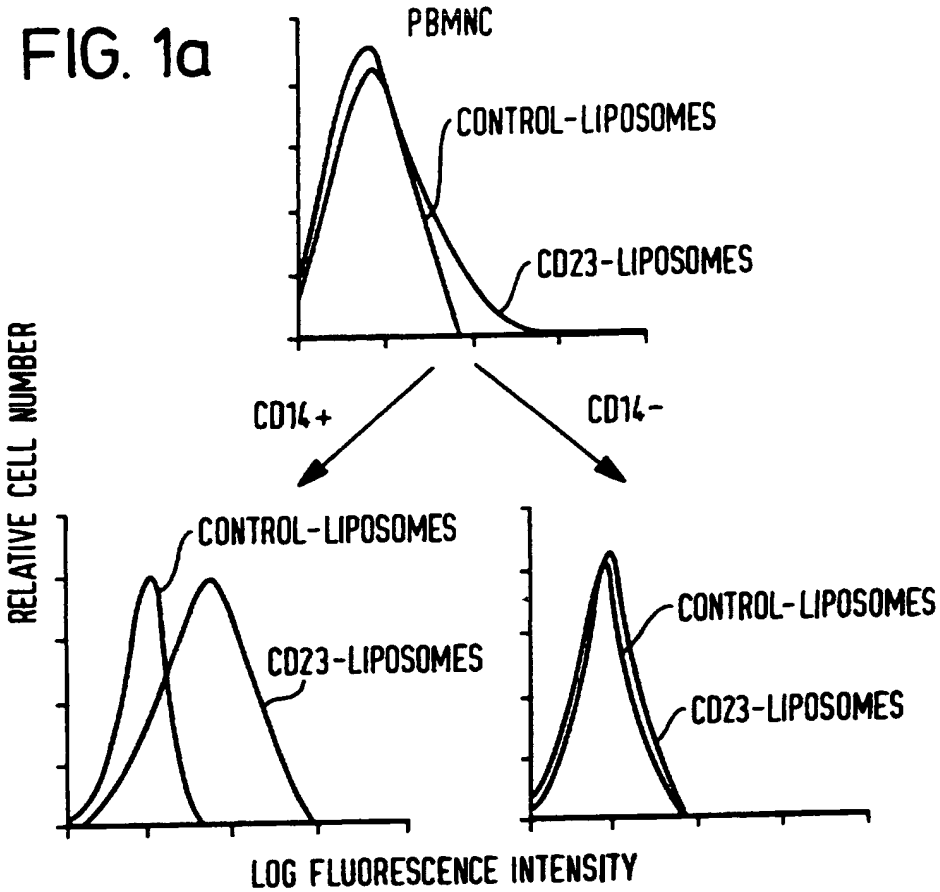

PBMNC
CONTROL-LIPOSOMES
CD23-LIPOSOMES
CD14+
CD14-
RELATIVE CELL NUMBER
CONTROL-LIPOSOMES
CD23-LIPOSOMES
CONTROL-LIPOSOMES
CD23-LIPOSOMES
LOG FLUORESCENCE INTENSITY

FIG. 1b

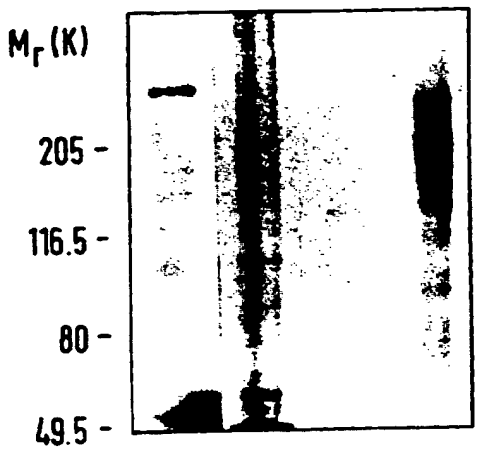
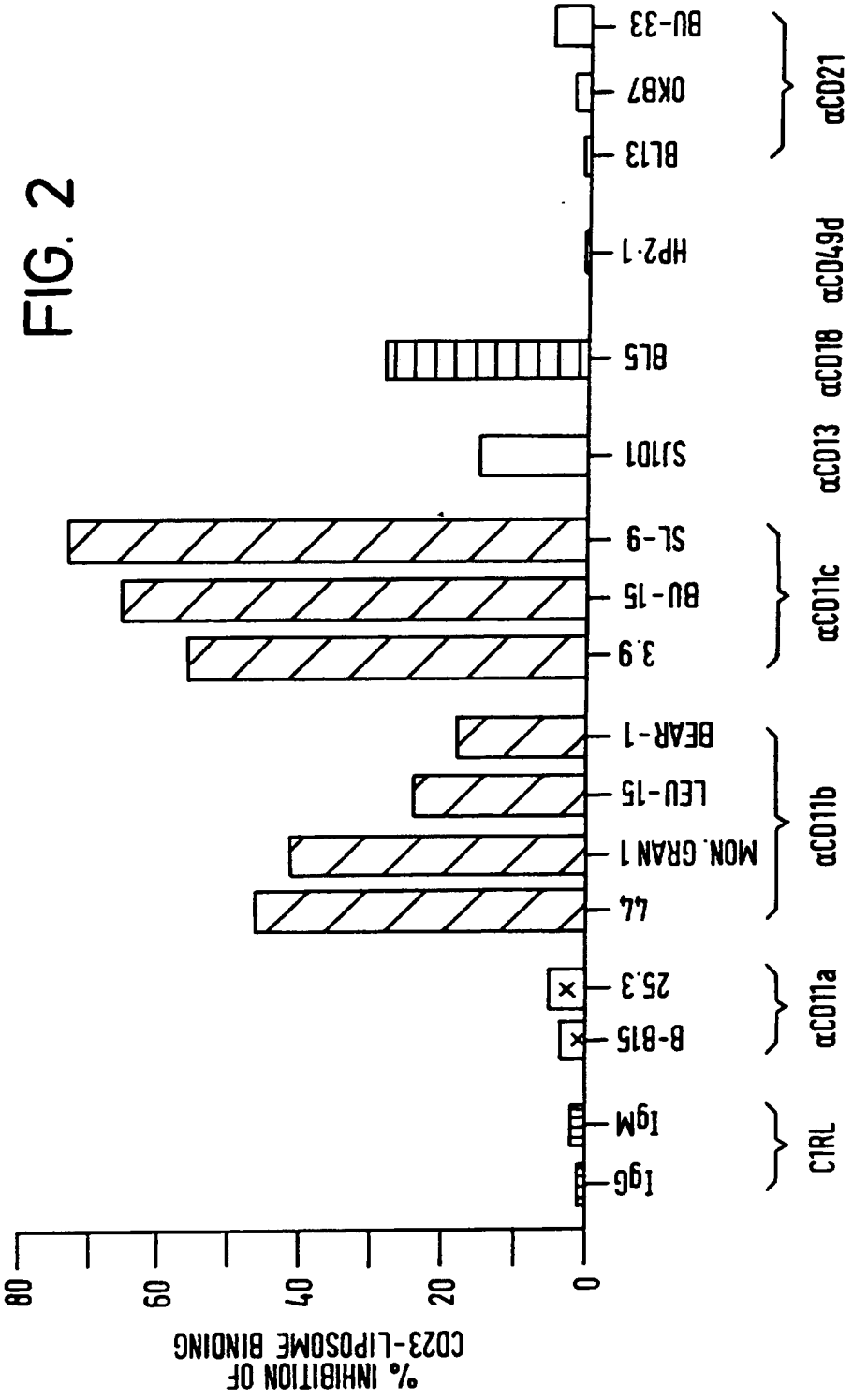

M_r (K)
205 -
116.5 -
80 -
49.5 -

FIG. 2


% INHIBITION OF
CD23-LIPOSOME BINDING
0
20
40
60
80
IgG
IgM
C1RL
B-B15
25.3
αCD11a
44
MON. GRAN 1
LEU-15
BEAR-1
αCD11b
3.9
BU-15
SL-9
αCD11c
SJ1D1
αCD13
BL5
αCD18
HP2.1
αCD49d
BL13
OKB7
BU-33
αCD21

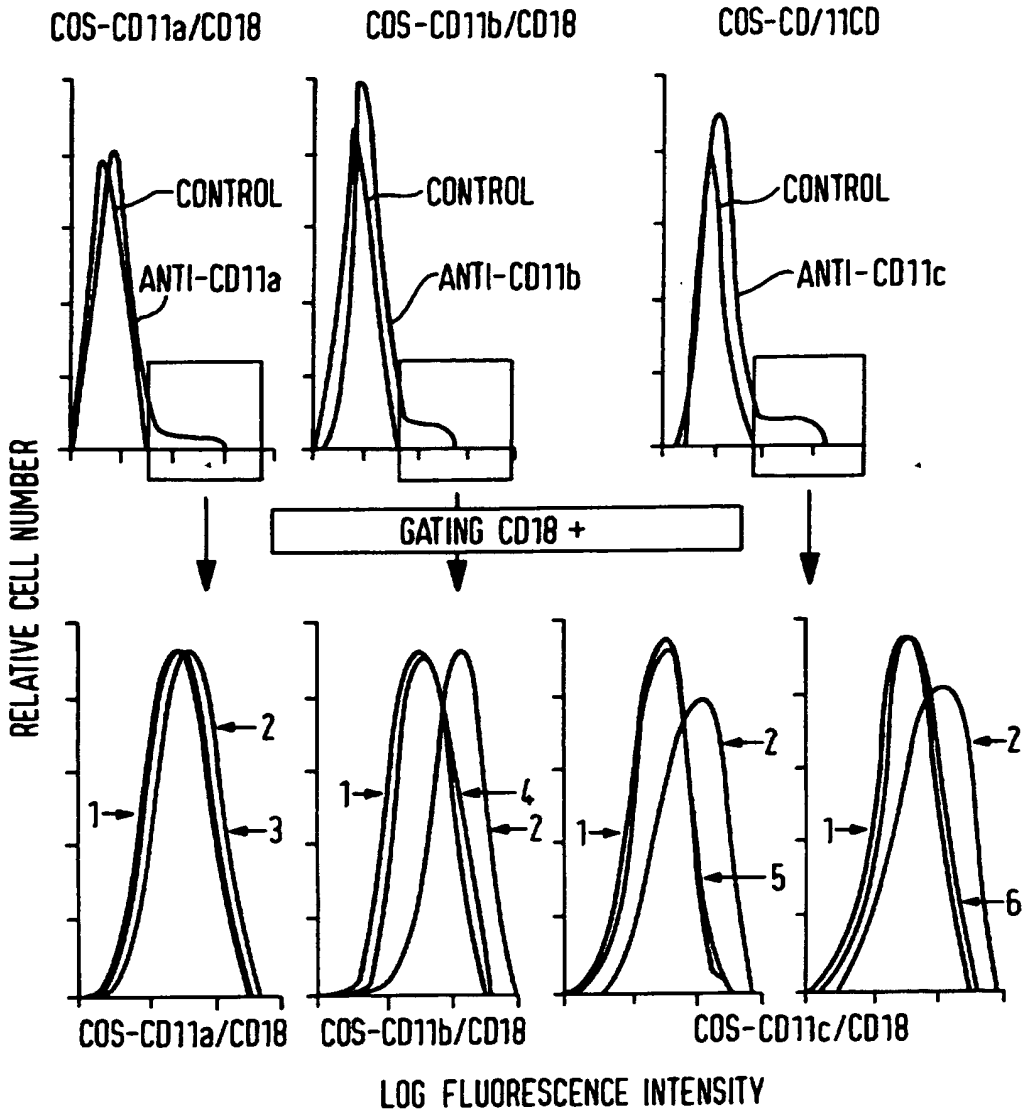

COS-CD11a/CD18
COS-CD11b/CD18
COS-CD/11CD
CONTROL
ANTI-CD11a
CONTROL
ANTI-CD11b
CONTROL
ANTI-CD11c
GATING CD18 +
RELATIVE CELL NUMBER
1
2
3
1
4
2
1
2
5
1
2
6
COS-CD11a/CD18
COS-CD11b/CD18
COS-CD11c/CD18
LOG FLUORESCENCE INTENSITY

FIG. 3

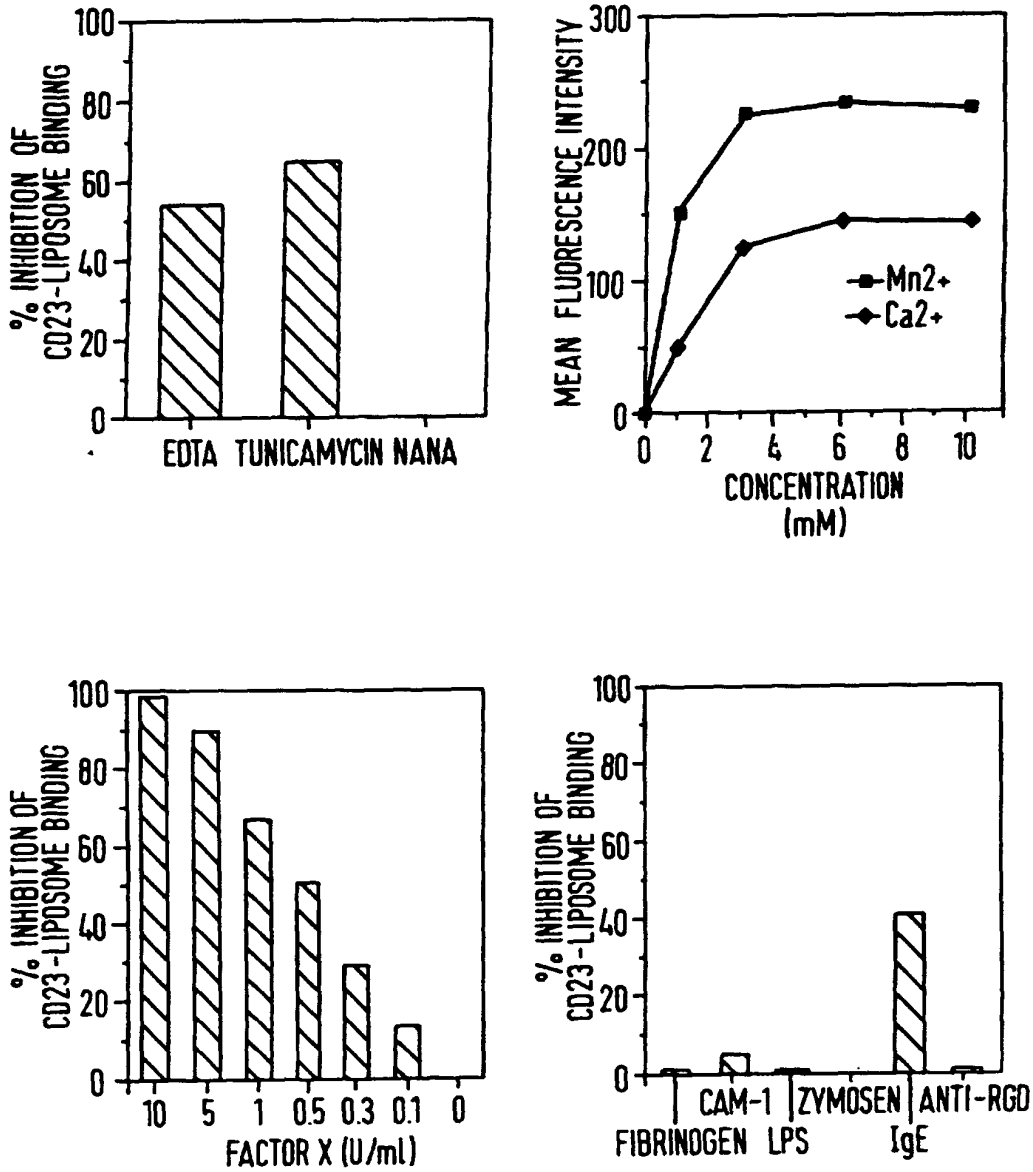

% INHIBITION OF CD23-LIPOSOME BINDING
100
80
60
40
20
0
EDTA
TUNICAMYCIN
NANA
MEAN FLUORESCENCE INTENSITY
300
200
100
0
Mn2+
Ca2+
0
2
4
6
8
10
CONCENTRATION (mM)
% INHIBITION OF CD23-LIPOSOME BINDING
10
5
1
0.5
0.3
0.1
0
FACTOR X (U/ml)
% INHIBITION OF CD23-LIPOSOME BINDING
CAM-1
ZYMOSEN
ANTI-RGD
FIBRINOGEN
LPS
IgE

FIG. 4

FIG. 5a

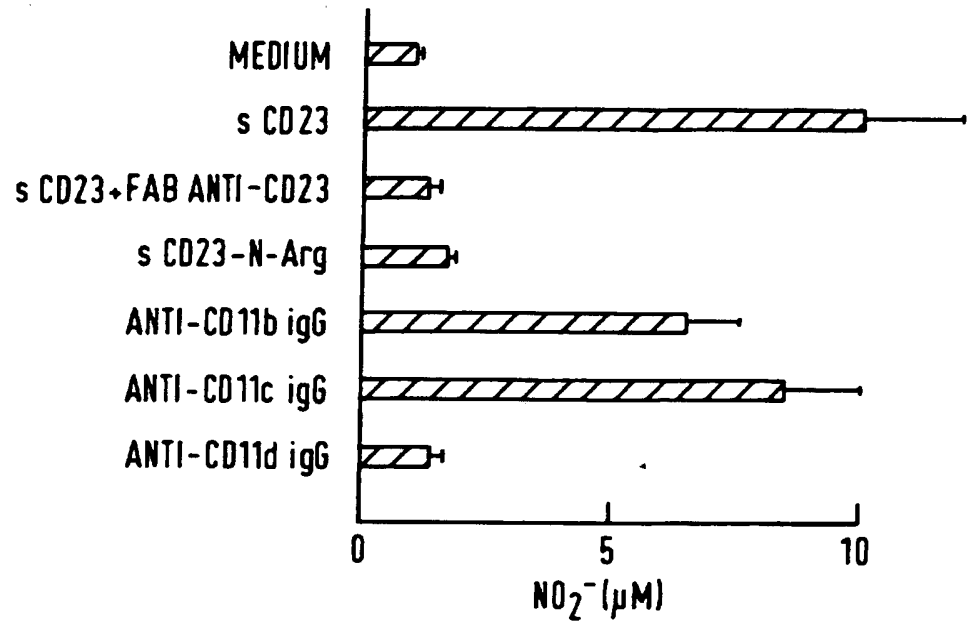

MEDIUM
s CD23
s CD23+FAB ANTI-CD23
s CD23-N-Arg
ANTI-CD11b igG
ANTI-CD11c igG
ANTI-CD11d igG
0
5
10
NO_2^- (μM)

FIG. 5b

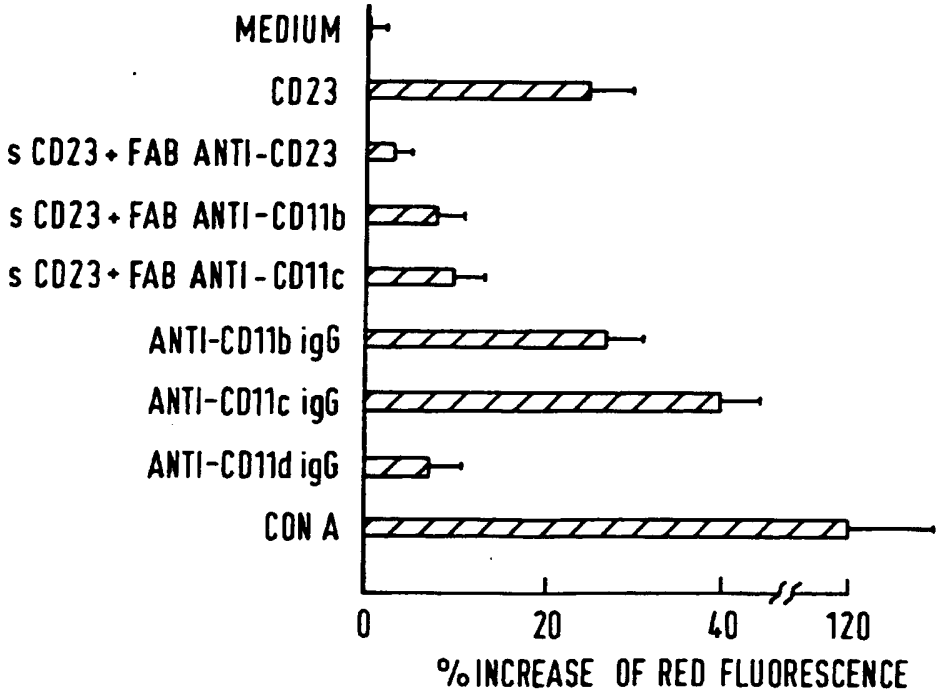

MEDIUM
CD23
s CD23+FAB ANTI-CD23
s CD23+FAB ANTI-CD11b
s CD23+FAB ANTI-CD11c
ANTI-CD11b igG
ANTI-CD11c igG
ANTI-CD11d igG
CON A
0
20
40
120
% INCREASE OF RED FLUORESCENCE

FIG. 6

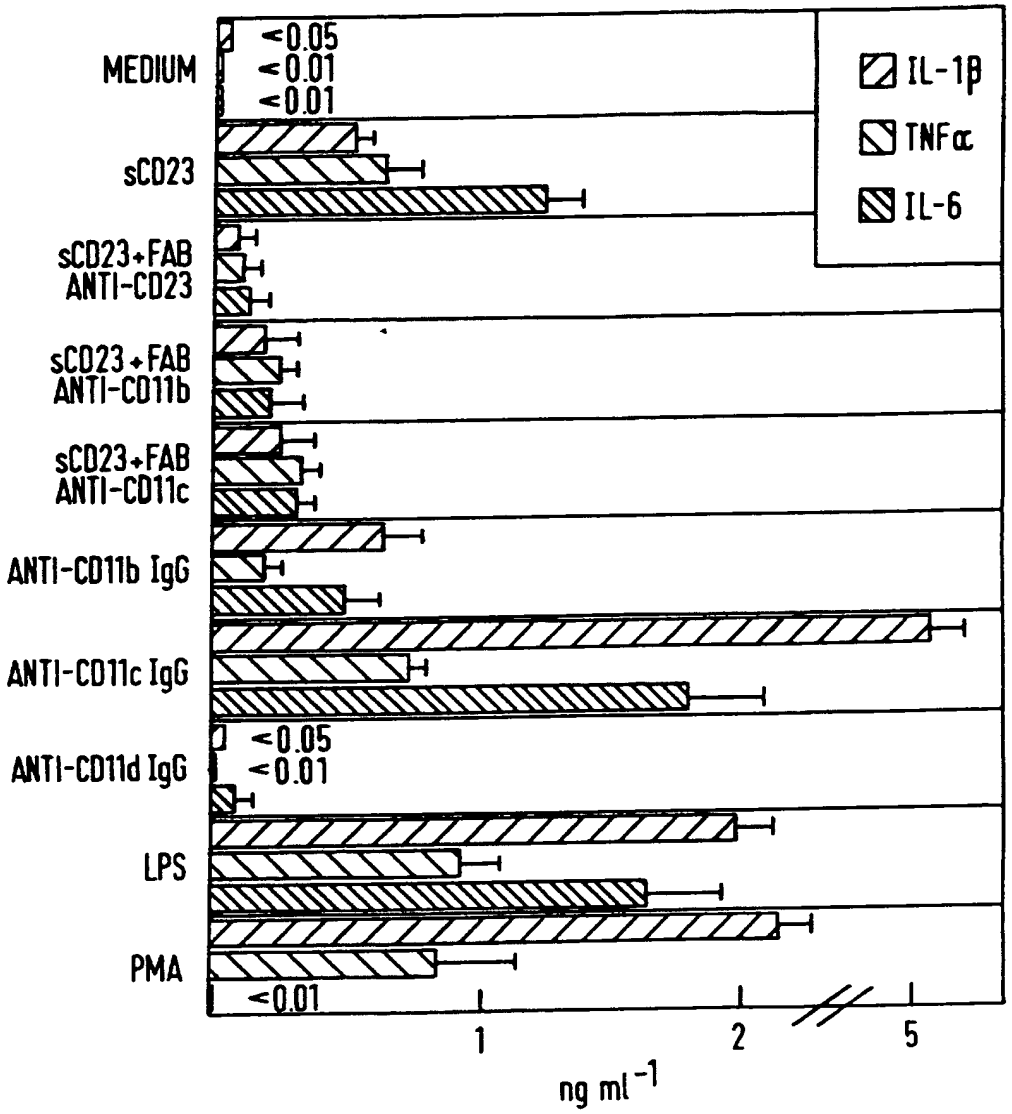

MEDIUM
sCD23
sCD23+FAB ANTI-CD23
sCD23+FAB ANTI-CD11b
sCD23+FAB ANTI-CD11c
ANTI-CD11b IgG
ANTI-CD11c IgG
ANTI-CD11d IgG
LPS
PMA
<0.05
<0.01
<0.01
<0.05
<0.01
<0.01
IL-1β
TNFα
IL-6
1
2
5
ng ml-1

FIG. 7a

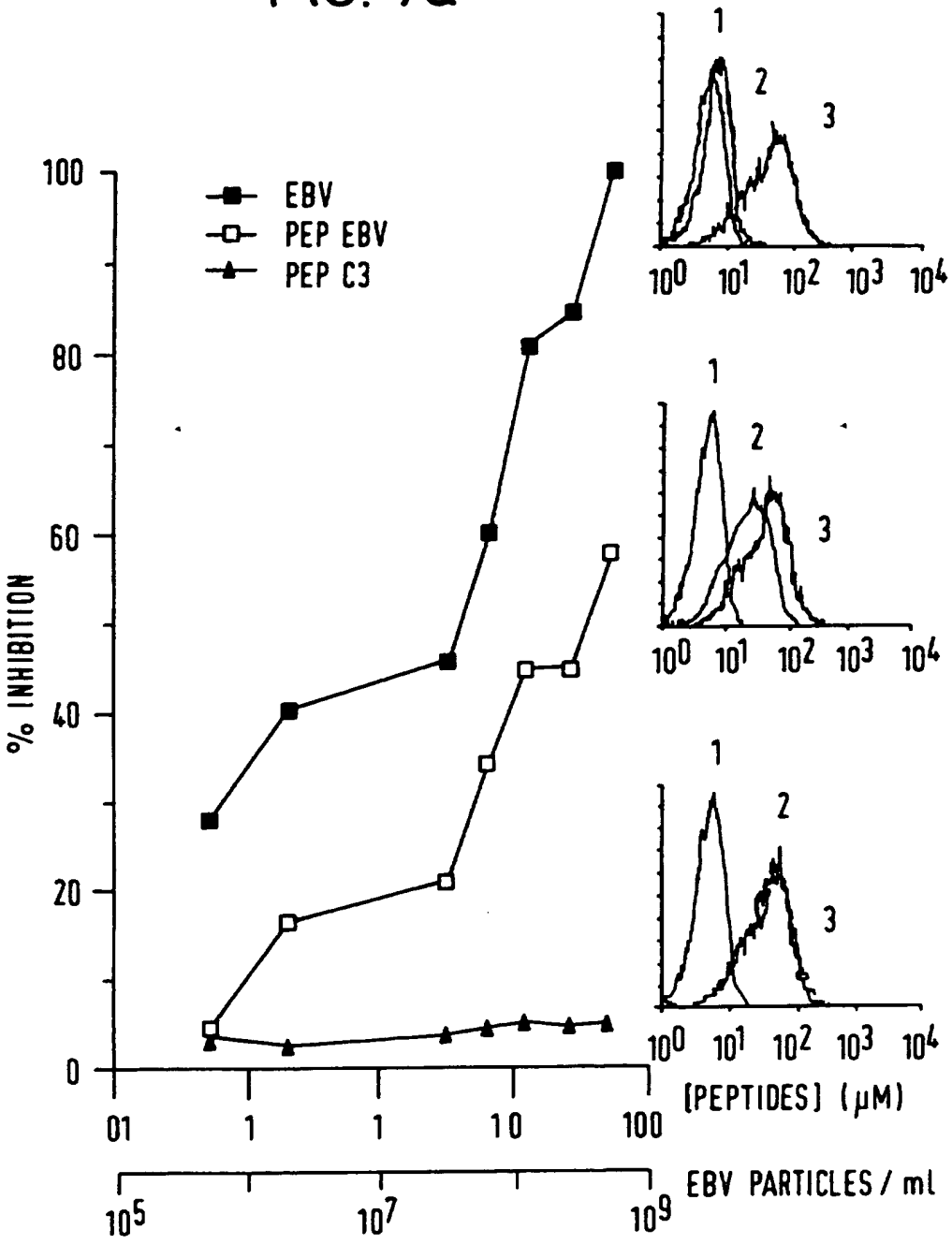

EBV
PEP EBV
PEP C3
% INHIBITION
100
80
60
40
20
0
01
1
1
10
100
[PEPTIDES] (μM)
10^5
10^7
10^9
EBV PARTICLES / ml
1
2
3
10^0
10^1
10^2
10^3
10^4

FIG. 7b

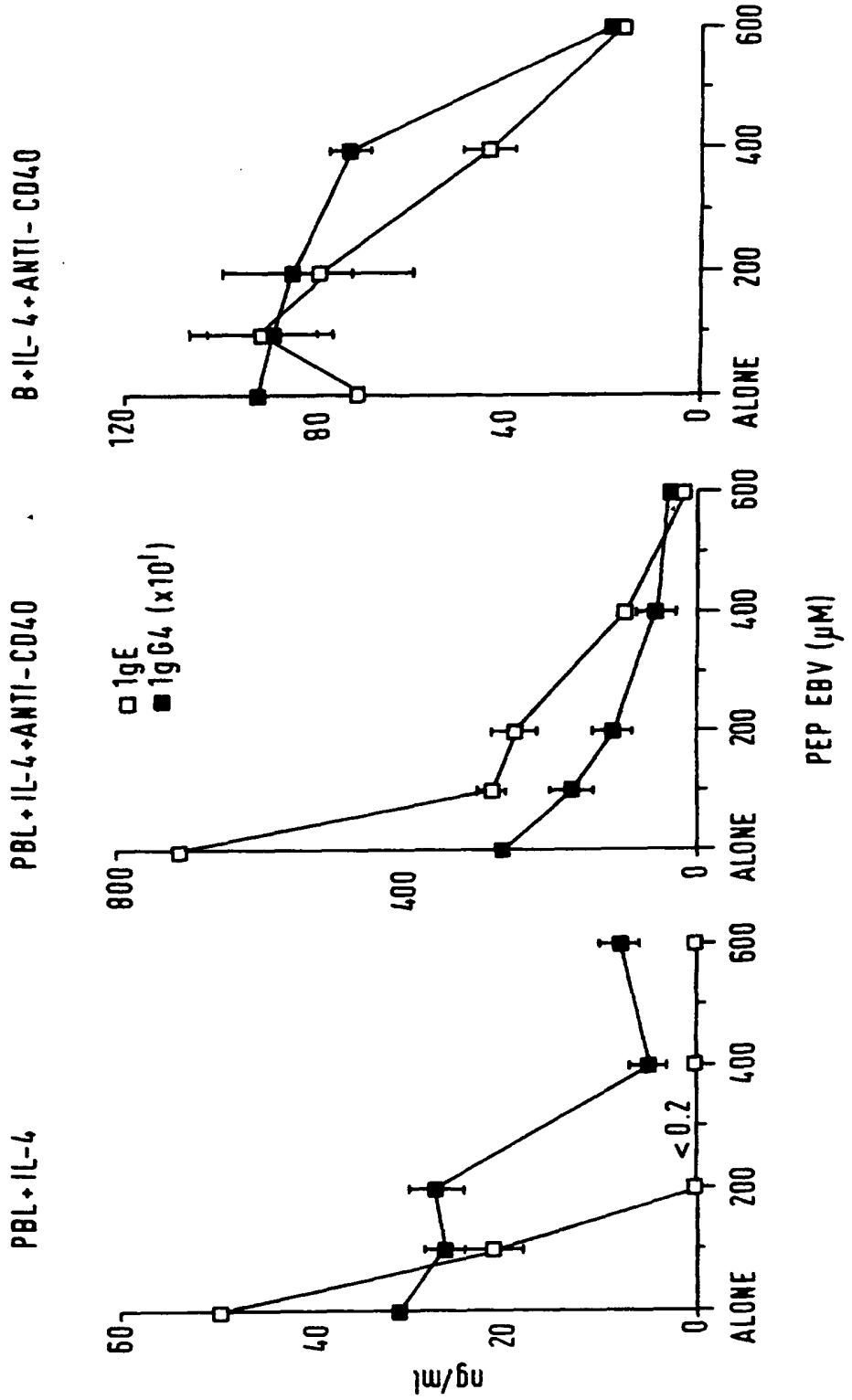

PBL + IL-4
PBL + IL-4 + ANTI-CD40
B + IL-4 + ANTI-CD40
ng/ml
□ IgE
■ IgG4 (x10¹)
PEP EBV (μM)
ALONE
< 0.2
200
400
600
0
20
40
60
80
120
800

FIG. 7c

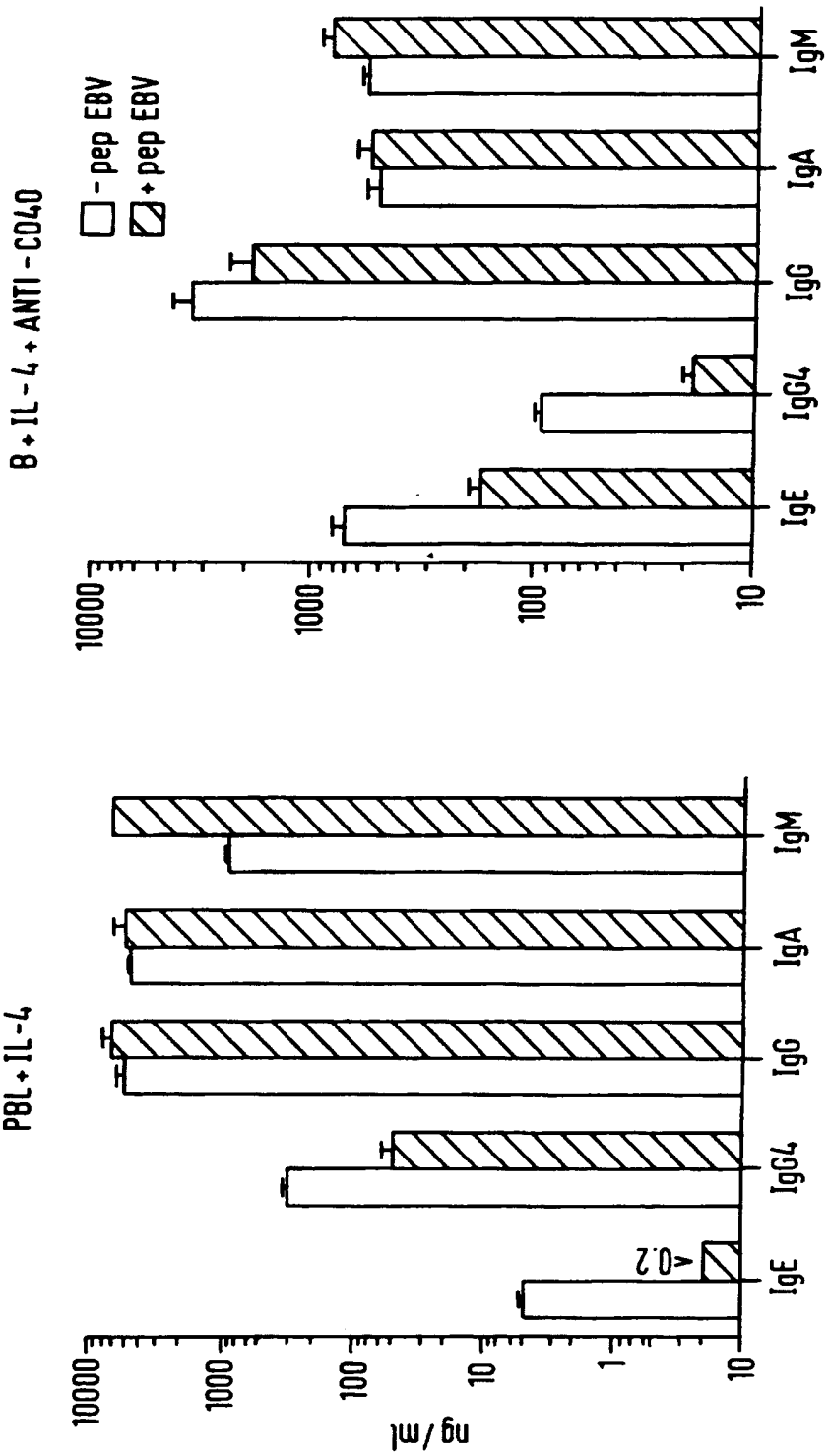

B + IL-4 + ANTI-CD40
- pep EBV
+ pep EBV
IgM
IgA
IgG
IgG4
IgE
10000
1000
100
10
PBL + IL-4
<0.2
1
ng/ml

FIG. 7d

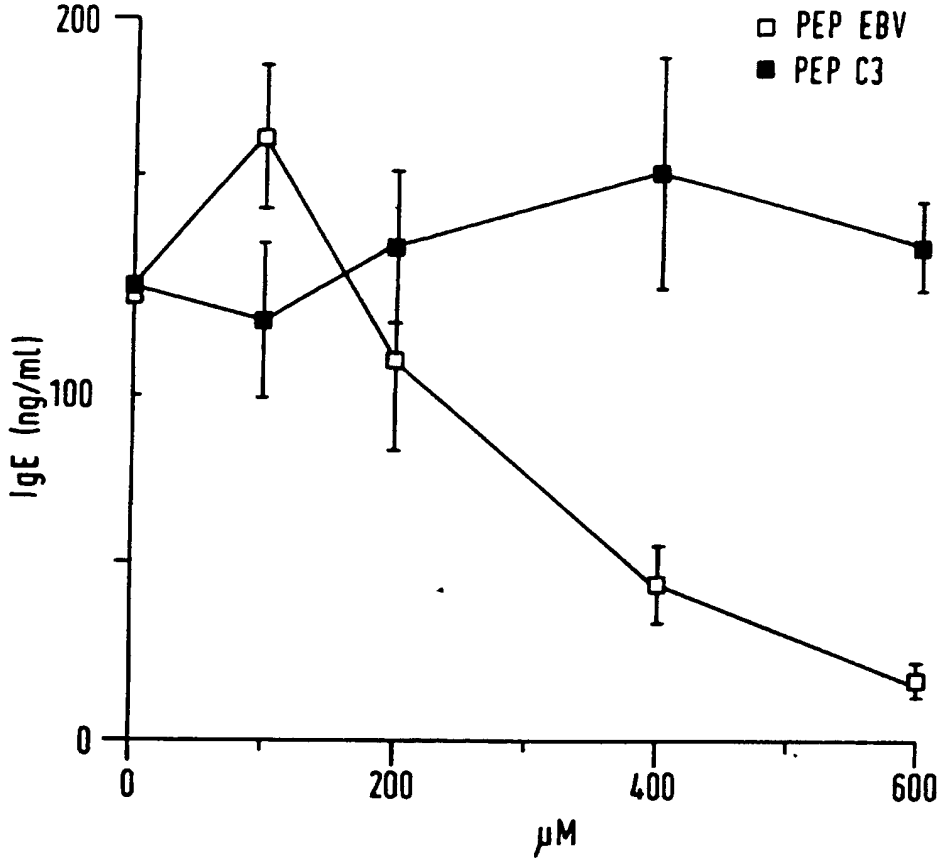
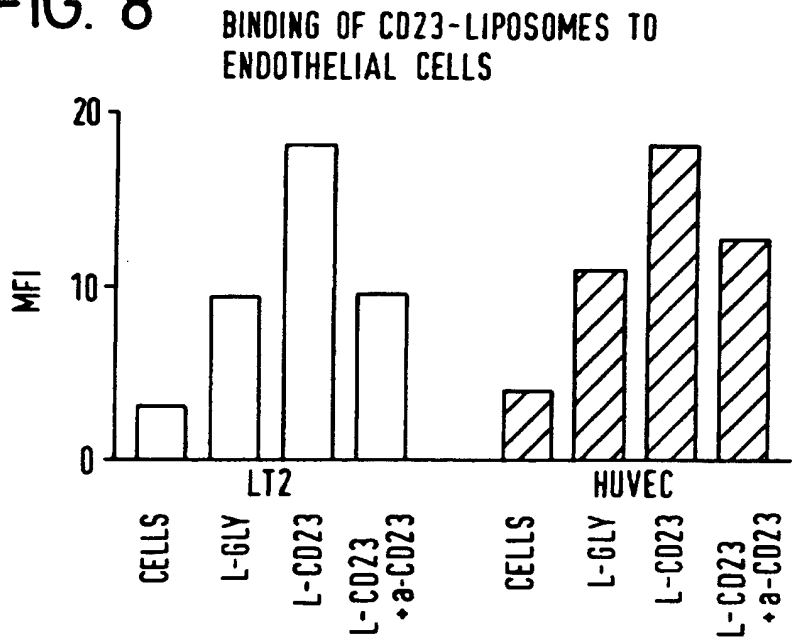

B + IL-4 + ANTI-CD40
PEP EBV
PEP C3
IgE (ng/ml)
200
100
0
0
200
400
600
μM

FIG. 8


BINDING OF CD23-LIPOSOMES TO ENDOTHELIAL CELLS
MFI
20
10
0
LT2
HUVEC
CELLS
L-GLY
L-CD23
L-CD23 + a-CD23
CELLS
L-GLY
L-CD23
L-CD23 + a-CD23

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 95/04110

A. CLASSIFICATION F SUBJECT MATTER
IPC 6 C07K16/28 C07K16/46 C07K14/05 C07K14/745 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AMERICAN JOURNAL OF PHYSIOLOGY, vol. 264, no. 4 part 2, April 1993 BETHESDA, MD, USA, pages F715-F721, XP 000562764 X. WU ET AL. 'Attenuation of immune-mediated glomerulonephritis with an anti-CD11b monoclonal antibody.' see abstract ---	1,2,7,8, 11,14-16
X	AMERICAN REVIEW OF RESPIRATORY DISEASE, vol. 147, no. 2, February 1993 USA, pages 435-441, P. PIGUET ET AL. 'Effective treatment of the pulmonary fibrosis elicited in mice by bleomycin or silica with anti-CD11 antibodies.' see abstract --- -/--	1,2,7,8, 11,14-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 1996	19.03.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 358 130 (O. GÖTZE) 14 March 1990 see page 4, line 31 - page 5, line 28 see claims 6-14	1,2,7-16
X	WO,A,90 04176 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 19 April 1990 see the whole document	1,4-7, 14-16
X	WO,A,93 02108 (IDEC PHARMACEUTICALS CORPORATION) 4 February 1993 cited in the application see page 37, line 25 - page 41, line 14 see claims	1-3,7-16
X	WO,A,93 09803 (THE SCRIPPS RESEARCH INSTITUTE) 27 May 1993 see the whole document	1,2,4, 8-16
X	WO,A,94 08620 (CENTER FOR BLOOD RESEARCH) 28 April 1994 see examples see claims	1,2,8,9, 11,12, 14-16
X	WO,A,92 01464 (THE SCRIPPS RESEARCH INSTITUTE) 6 February 1992 see page 37, line 7 - page 41, line 32 see examples see claims	1,8-16
X	WO,A,89 04174 (H. ROSEN ET AL.) 18 May 1989 see examples see claims	1,2,8-16
X	EP,A,0 321 842 (T. KISHIMOTO) 28 June 1989 see the whole document	1,8-16
A	NATURE, vol. 358, no. 6386, 6 August 1992 LONDON, GB, pages 505-507, J. AUBRY ET AL. 'CD21 is a ligand for CD23 and regulates IgE production.' cited in the application see abstract	1,2,4-16
A	NATURE, vol. 310, 19 July 1984 LONDON, GB, pages 207-211, R. BAER ET AL. 'DNA sequence and expression of the B95-8 Epstein-Barr virus genome.' see the whole document	4-6

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 23, no. 7, July 1993 WEINHEIM, GERMANY, pages 1739-1742, B. HEYMAN ET AL. 'In vivo enhancement of the specific antibody response via the low-affinity receptor for IgE.' see abstract	7
P,X	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 24, no. 12, December 1994 WEINHEIM, GERMANY, pages 2982-2986, I. GROSJEAN ET AL. 'CD23/CD21 interaction is required for presentation of soluble protein antigen by lymphoblastoid B cell lines to specific CD4+ T cell clones.' see abstract see page 2986, left column, line 5 - line 18	1,2,7-9, 11,12, 14-16
P,X	IMMUNITY, vol. 3, no. 1, July 1995 USA, pages 119-125, S. LECOANET-HENCHOZ ET AL. 'CD23 regulates monocyte activation through a novel interaction with the adhesion molecules CD11b-CD18 and CD11c-CD18.' see abstract see page 123, right column, line 3 - line 22	1,2,7-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 95/04110

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 16
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 16 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter nal Application No
PCT/EP 95/04110

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-358130	14-03-90	DE-A-	3830271	15-03-90
WO-A-9004176	19-04-90	AU-B-	4341189	01-05-90
		EP-A-	0436626	17-07-91
		GR-A-	89100631	29-11-90
WO-A-9302108	04-02-93	AP-A-	307	31-01-94
		AU-B-	2425592	23-02-93
		BG-A-	98411	28-02-95
		BR-A-	9206313	11-04-95
		CA-A-	2114015	04-02-93
		CZ-A-	9400149	13-07-94
		EP-A-	0605442	13-07-94
		FI-A-	940336	10-03-94
		HU-A-	70272	28-09-95
		JP-T-	6509708	02-11-94
		NO-A-	940219	25-03-94
		NZ-A-	243706	26-08-94
		OA-A-	9879	15-09-94
		PT-A-	100735	29-10-93
		SK-A-	8894	07-09-94
WO-A-9309803	27-05-93	AU-B-	3221793	15-06-93
WO-A-9408620	28-04-94	AU-B-	5442394	09-05-94
		EP-A-	0670734	13-09-95
WO-A-9201464	06-02-92	US-A-	5473051	05-12-95
		JP-T-	5509322	22-12-93
		AU-B-	8425191	18-02-92
		CA-A-	2087169	20-01-92
		EP-A-	0540675	12-05-93
WO-A-8904174	18-05-89	EP-A-	0386059	12-09-90
		JP-T-	3501562	11-04-91
EP-A-321842	28-06-89	EP-A-	0321601	28-06-89
		EP-A-	0324879	26-07-89
		AU-B-	2738088	13-07-89
		JP-A-	2000443	05-01-90